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Crystallization of cytochrome P-450$_{scc}$ from bovine adrenocortical mitochondria

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Cytochrome P-450$_{scc}$ (P-450$_{scc}$), a cholesterol side-chain cleavage enzyme from bovine adrenocortical mitochondria, has been crystallized for the first time. Upon removal of glycerol from the solution of the native enzyme complexed with pyridoxal 5'-phosphate (PLP) by microdialysis against distilled water, reddish and planar crystals appeared. The crystals of native P-450$_{scc}$ were also obtained by the same procedure. We identified the crystals as the P-450$_{scc}$-PLP complex or native P-450$_{scc}$ by absorption spectroscopy and SDS-polyacrylamide gel electrophoresis, and characterized them under a polarization microscope.

Cytochrome P-450$_{scc}$; Membrane protein; Crystallization; Pyridoxal 5'-phosphate; Glycerol; Polarization microscope

1. INTRODUCTION

Cytochrome P-450$_{scc}$ (P-450$_{scc}$), a cholesterol side-chain cleavage enzyme, which is located in the inner mitochondrial membrane, catalyzes the conversion of cholesterol to pregnenolone [1,2], the initial rate-limiting reaction in the synthesis of various steroid hormones in the adrenal cortex. Of various P-450 isozymes, only P-450$_{cam}$, the soluble enzyme from the bacterium, *Pseudomonas putida*, has been crystallized using ammonium sulfate as a precipitant [3] and its high-resolution crystal structure has been recently determined by Poulos et al. [4]. No animal isozymes, however, have been crystallized yet.

Here we report the crystallization of P-450$_{scc}$ from the mammalian tissue, the bovine adrenal cortex, for the first time, which may be the first step toward the X-ray crystallographic study of an animal P-450 isozyme.

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Abbreviations: P-450$_{scc}$, cytochrome P-450$_{scc}$; PLP, pyridoxal 5'-phosphate; P-450$_{scc}$-PLP complex, covalent complex of cytochrome P-450$_{scc}$ with pyridoxal 5'-phosphate

2. METHODS AND RESULTS

2.1. *Preparation of the enzyme*

P-450$_{scc}$ was purified from bovine adrenocortical mitochondria by DEAE-cellulose, hydroxyapatite gel and adrenodoxin-Sepharose 4B column chromatographies as previously described by Tsubaki et al. [5]. The nonionic detergent, Emulgen 913, was removed by exhaustive washing of the enzyme on the affinity column with a buffer containing the ionic detergent, sodium cholate. Sodium cholate was removed by dialysis against 20 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1 mM EDTA (buffer A). All procedures for purification were performed in the presence of 20% (v/v) glycerol according to the method of Ichikawa and Yamano [6]. Protein concentration was determined by the biuret method. P-450 content was estimated by using $129.1 \times 10^3\ M^{-1} \cdot cm^{-1}$ as the molecular extinction coefficient at 448 nm in the absolute spectrum of the sodium dithionite-reduced CO complex [5]. The purified sample had a heme content of 19.0 nmol/mg protein and showed a single protein staining band with a molecular mass of 51 kDa on SDS-polyacrylamide gel electrophoresis.

P-450$_{scc}$ complexed with cholesterol in buffer A was mixed with 10 mM PLP dissolved in buffer A to final concentrations of 10 mg protein/ml and 2 mM PLP. After the mixture was incubated at 25°C for 90 min, it was reduced with $NaBH_4$ to attain the irreversible binding of PLP to the lysine residues of P-450$_{scc}$ and was then subjected to dialysis against buffer A.

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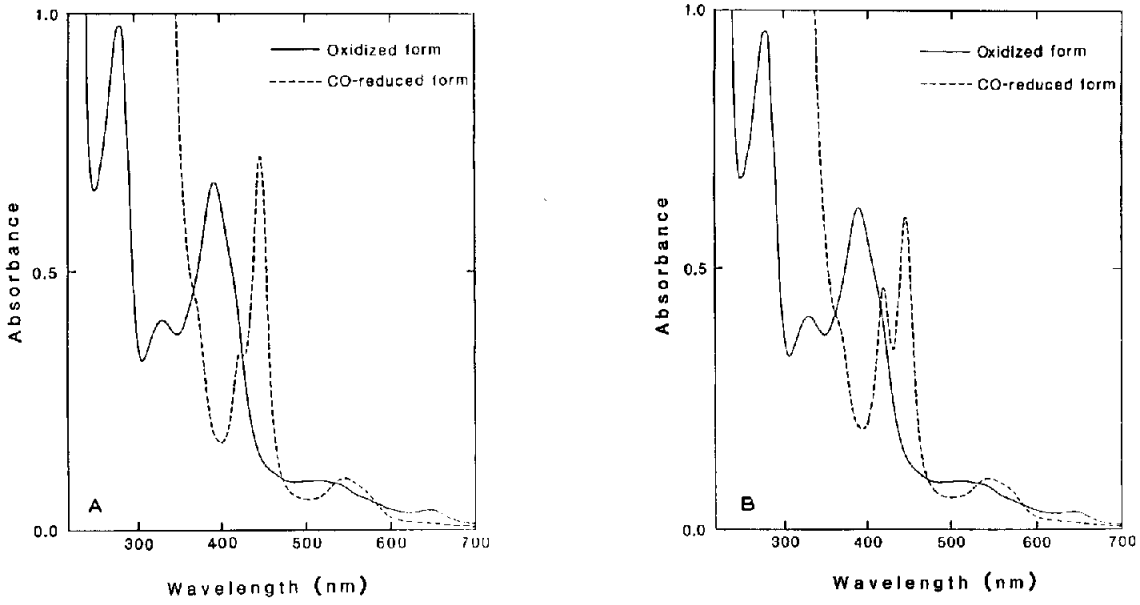


Fig.1. Absorption spectra of the P-450$_{scc}$-PLP complex. (——) Oxidized form; (– – –) sodium dithionite-reduced CO form. (A) Absorption spectra of the P-450$_{scc}$-PLP complex before crystallization. (B) Absorption spectra of the crystalline P-450$_{scc}$-PLP complex.

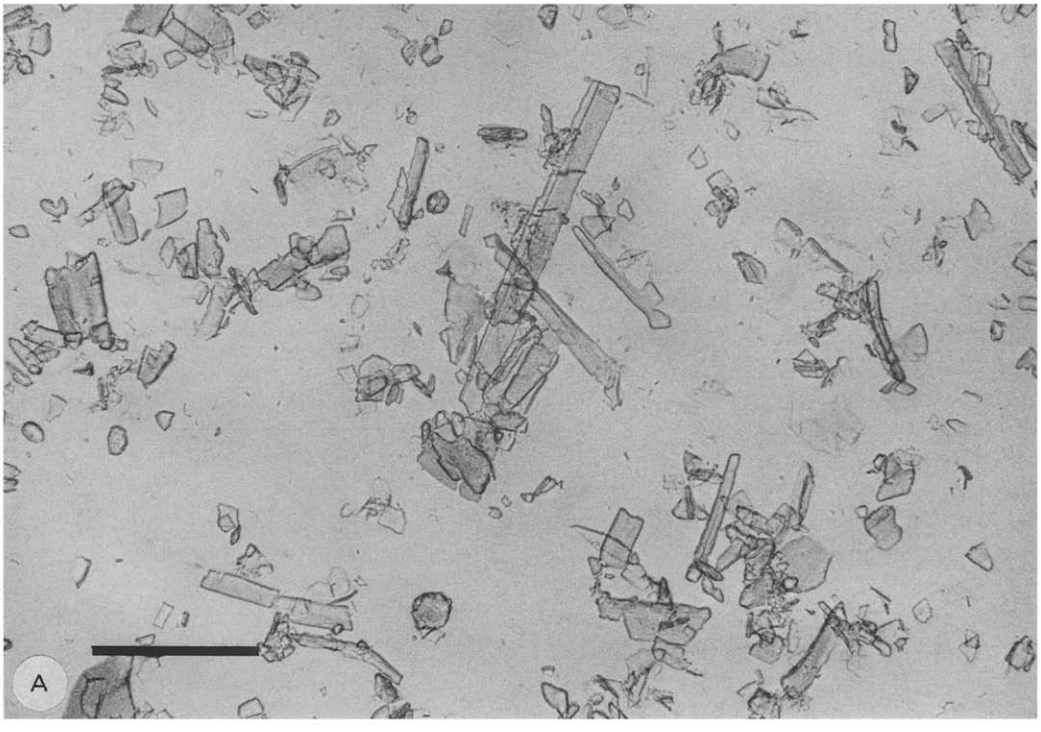
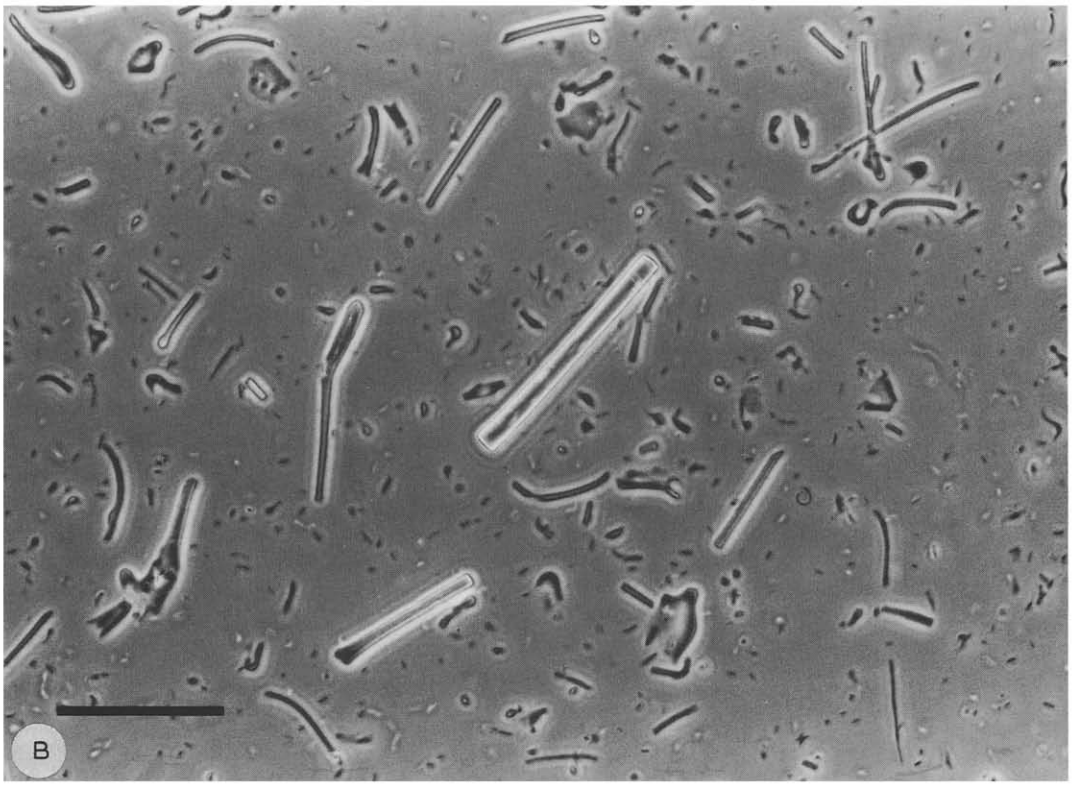


Fig.2. For legend see p.34.



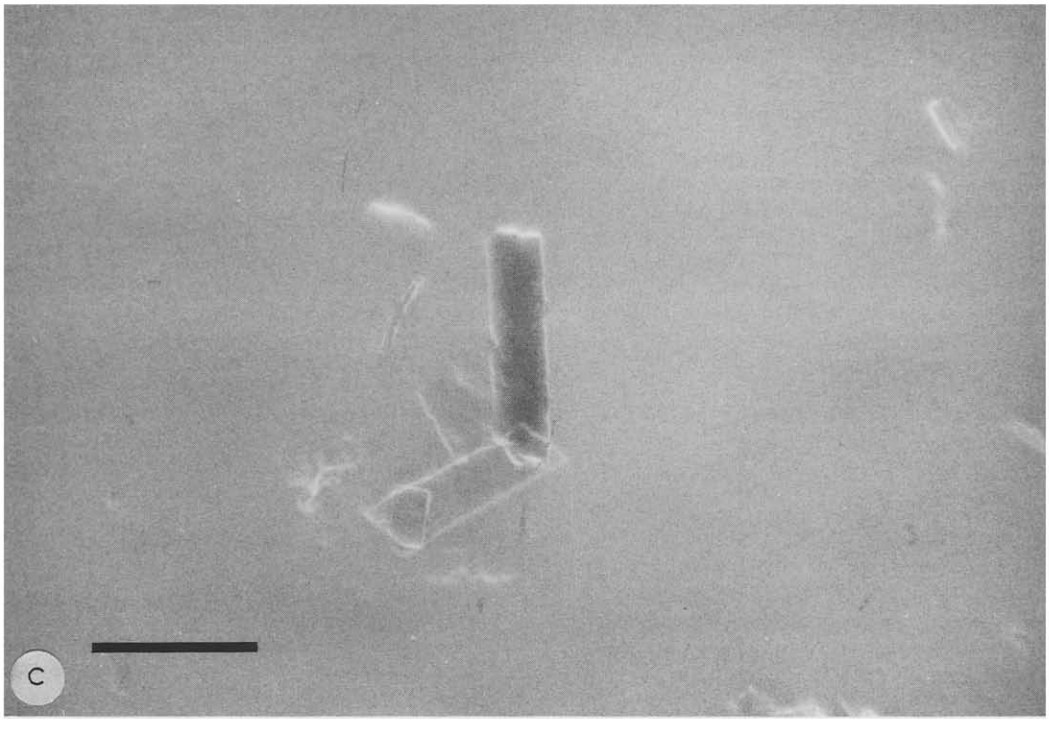


Fig.2. (Contd).

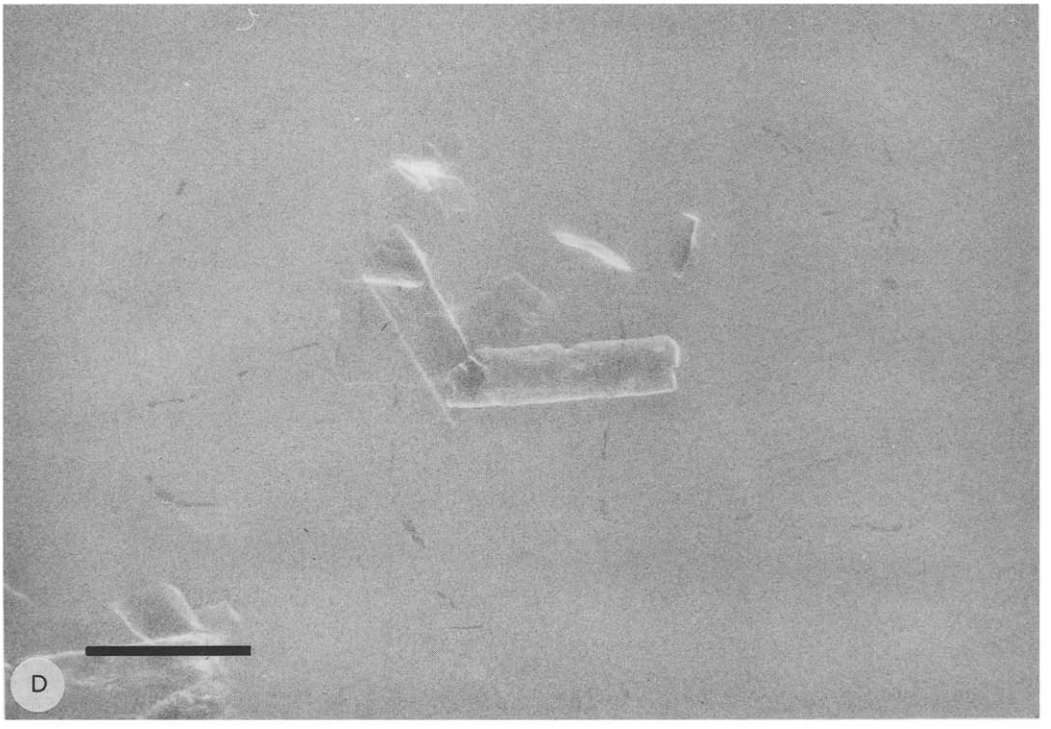


Fig.2. (A) Crystals of the P-450$_{scc}$-PLP complex observed under a microscope in bright-field. Bar = 50 μm. (B) Crystals of native P-450$_{scc}$ observed under a phase-contrast microscope. Bar = 50 μm. (C) A type B crystal (the largest one) of the P-450$_{scc}$-PLP complex. The picture was taken with light polarized parallel to the short axis of the crystal. Another large crystal crossed with the type B crystal is a type A crystal. Bar = 50 μm. (D) Same crystals as in C, but taken with light polarized parallel to the long axis of the type B crystal. Bar = 50 μm.

The resulting sample showed a high-spin character in the absorption spectrum due to binding of cholesterol to the substrate binding site. In addition, a new absorption band at 325 nm, characteristic of pyridoxal 5′-phosphate-ϵ-lysine, was observed (fig.1A). There was no formation of P-420 form upon this treatment (fig.1A) and the modified enzyme became much stabler than native enzyme on spectroscopic criteria. (That is, during standing in the CO-reduced form at room temperature, the native enzyme became the P-420 form very soon, whereas the PLP-modified enzyme took a much longer time to undergo this transformation.) This sample was used initially for the crystallization experiment.

2.2. *Crystallization*

A microdialysis method was adopted. A droplet of the P-450$_{scc}$-PLP complex (10 mg/ml) in buffer A was dialyzed at 4°C without stirring against 500 ml of distilled water, whose pH was previously adjusted to 5.5–6.0 with 1 mM NaOH. After 1 week of dialysis, reddish and planar crystals were seen under a light microscope (fig.2A).

We also crystallized native P-450$_{scc}$ by the same procedure except that pH of the dialysis medium was adjusted to 7.0–8.0 (fig.2B). Reddish and thin column-shaped crystals were formed after 1 week of dialysis. Removal of glycerol from the enzyme solution during the dialysis was essential for the formation of the crystals in each case.

2.3. *Identification of crystals as P-450$_{scc}$*

The crystals grown from the P-450$_{scc}$-PLP complex were redissolved in buffer A and were analyzed by absorption spectroscopy and SDS-polyacrylamide gel electrophoresis. The absorption spectrum of the oxidized form showed a close similarity to that of the complex before the crystallization (fig.1B). The absorption spectrum of the CO-reduced form indicates a slight increase of P-420 form upon the crystallization. This may be due to a partial denaturation and precipitation of the enzyme during the dialysis. Upon SDS-polyacrylamide gel electrophoresis, the crystalline complex co-migrated with the complex before the crystallization (fig.3). Similar analyses also established the identity of the crystals grown from the untreated enzyme as native P-450$_{scc}$ (not shown).

2.4. *Polarization microscope study*

Both the native and the PLP-complex crystals were subjected to analyses using a polarization microscope. We applied the plane-polarized light of various directions to a crystal and measured the optimal exposure time for taking a photomicrograph of a crystal by utilizing the built-in exposure

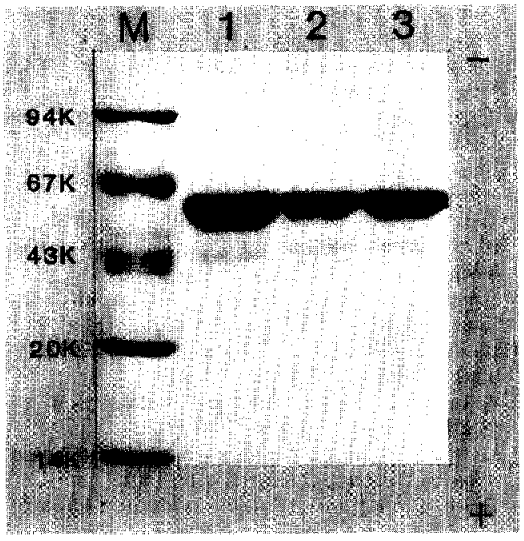


Fig.3. SDS-polyacrylamide gel electrophoretic analysis of the P-450$_{scc}$-PLP complex. Lane M contains standard molecular mass markers: phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14400). Lanes: 1, 20 μg untreated P-450$_{scc}$; 2, 20 μg the P-450$_{scc}$-PLP complex before crystallization; 3, 20 μg the crystalline P-450$_{scc}$-PLP complex.

meter of the camera system for automatic photomicrography (Nikon, UFX-2-35A). To be concrete, the optimal exposure time was measured with the measuring spot, which occupied 1% of the field of a microscope and was within a crystal at × 400 magnification. The angle dependence of the optimal exposure time for a crystal can be thought to reflect the angle dependence of the absorbance of the crystal against the plane-polarized light.

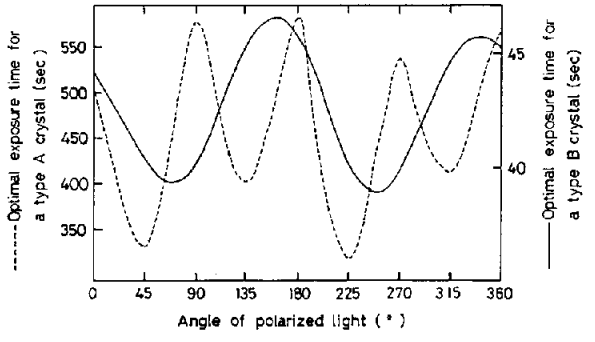


Fig.4. Dependence of the optimal exposure time of a single type A (– – –) and a type B (——) crystal on the direction of the polarized light. At an angle of 160° or 340° the plane-polarized light is parallel to the short axis of the type B crystal. At an angle of 90°, 180°, 270° or 360° the plane-polarized light is parallel to the long or short axis of the type A crystal. Measurements performed on five different single crystals of each type did not reveal differences in the periodicity of the curves, but there was a variation in the height of the curves.

As seen in fig.4, the P-450$_{scc}$-PLP complex crystals could be classified into two types by the dependence of the exposure time on the direction of the incident polarized light. One type of crystal (type A crystal) required the longest optimal exposure time when the polarized light was parallel to the long or short axis of the crystal and the shortest time when 45° with the axis, while another type (type B crystal) required the longest time when parallel to the short axis and the shortest time when parallel to the long axis (fig.2C and D). We also found the existence of two types of native P-450$_{scc}$ crystals, which showed the same patterns of dependence of the optimal exposure time on the direction of the polarized light as the two types of the P-450$_{scc}$-PLP complex crystals, respectively. These results indicate that both of type A and B crystals are single crystals.

3. DISCUSSION

P-450 isozymes have been found in bacteria [7,8], in yeast [9] and in mitochondria and microsomes of animal tissues [10–14]. Of these isozymes, only P-450$_{cam}$, the bacterial isozyme soluble in aqueous media without detergent has been crystallized using ammonium sulfate as a precipitant. On the other hand, the other P-450 isozymes are membrane proteins and use of the detergent is essential for their solubilization and purification. In the present study we crystallized P-450$_{scc}$ from bovine adrenocortical mitochondria. This is the first successful crystallization of the P-450 isozyme located in the membrane.

Since Ichikawa and Yamano [6] found that addition of glycerol prevented mammalian P-450 from converting to P-420, purification of mammalian P-450 isozymes has been generally performed in the presence of 20% (v/v) glycerol. In the present study, however, we found that removal of glycerol from the P-450$_{scc}$ solution resulted in the crystallization of the enzyme. For the crystallization of native P-450$_{scc}$, the optimum pH for the dialysis was 7.0–8.0; however, to crystallize the P-450$_{scc}$-PLP complex successfully, pH of the dialysis medium should be 5.5–6.0. This may be due to the decrease of the isoelectric point of P-450$_{scc}$ caused by the binding of PLP to the lysine residues of the enzyme. P-450$_{scc}$ complexed with PLP was much stabler and crystallized more successfully than the native enzyme, probably because the covalent PLP modification of the enzyme increased binding affinity of cholesterol to the enzyme (Tsubaki et al., unpublished). The

crystallization of the complex or the native enzyme, however, occurs both in the presence and absence of substrate, cholesterol.

In conclusion, the crystallization of P-450$_{scc}$ opens a new door to the study of relationship between the function and the three-dimensional structure of membraneous P-450 isozymes.

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